

# A New Species of Genus *Microhyla* (Amphibia: Anura: Microhylidae) from Zhejiang Province, China

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**Abstract** We described a new species, *Microhyla beilunensis* sp. nov., from Zhejiang Province of China. Phylogenetic analyses based on the mitochondrial 12S, 16S and CO1 gene sequences suggested that the new taxon was distinctly separated from its congeners and closed to *M. mixtura* and *M. okinavensis*. Morphologically, the new species could be identified from its congeners except *M. mixtura* by several characters: (1) rudimentary webs on toe base; (2) absence of disks and dorsal median longitudinal grooves on finger tips; (3) presence of disks and dorsal median longitudinal grooves on toe tips. As well, the new species could be identified from topotype *M. mixtura* by the combination of characters: (1) apart from the stripes, bar-shaped and oval-shaped patterns, the rounded spots present on the dorsum of body and legs; (2) the outer metacarpal tubercles prominently larger than the inner one; (3) of males, the ratios of HW, IND, UEW and LAW to SVL of the new species were significantly larger than those of *M. mixtura* ( $P < 0.01$ ), and the ratios of SL, IOD, LAHL, HLL, TL, TFL and FL to SVL of the new species were significantly less than those of *M. mixtura* ( $P < 0.05$ ).

**Keywords** *Microhyla*, morphological comparison, new species, phylogenetic analysis, taxonomy, Zhejiang

## 1. Introduction

The genus *Microhyla* Tschudi, 1838 (Amphibia: Anura: Microhylidae) is widely distributed in Eastern, Southeastern and Southern Asia. It currently contains 41 species (Frost, 2018). As noted, 13 *Microhyla* species have been described in the past decade, of which, five species, *M. arboricola*, *M. darevskii*, *M. minuta*, *M. pineticola* and *M. pulchella* from Vietnam (Poyarkov *et al.*, 2014), three species, *M. mukhlesuri*, *M. mymensinghensis*, *M. nilphamariensis* from Bangladesh

(Hasan *et al.*, 2014; Howlader *et al.*, 2015), and the remaining five species, *M. malang* from Malaysia (Matsui, 2011), *M. orientalis* from Indonesia (Matsui *et al.*, 2013), *M. laterite* from India (Seshadri *et al.*, 2016), *M. mihintalei* from Sri Lanka (Wijayathilaka *et al.*, 2016), *M. taraiensis* from Nepal (Khatiwada *et al.*, 2017). Even so, molecular studies still indicated that several cryptic species in the genus remained to be described, especially in the species with a wide geographical range inhabiting varied habitats (Howlader *et al.*, 2015; Khatiwada *et al.*, 2017; Matsui *et al.*, 2011).

*Microhyla mixtura* Liu and Hu, 1966 is an endemic species to China, and was widely distributed in Shaanxi, Henan, Chongqing, Sichuan, Hubei, Guizhou, Anhui and Zhejiang provinces of China (Fei and Ye, 1982; Chen, 1991; Fei *et al.*, 2012). Nevertheless, many previous studies proposed that there were significant differences

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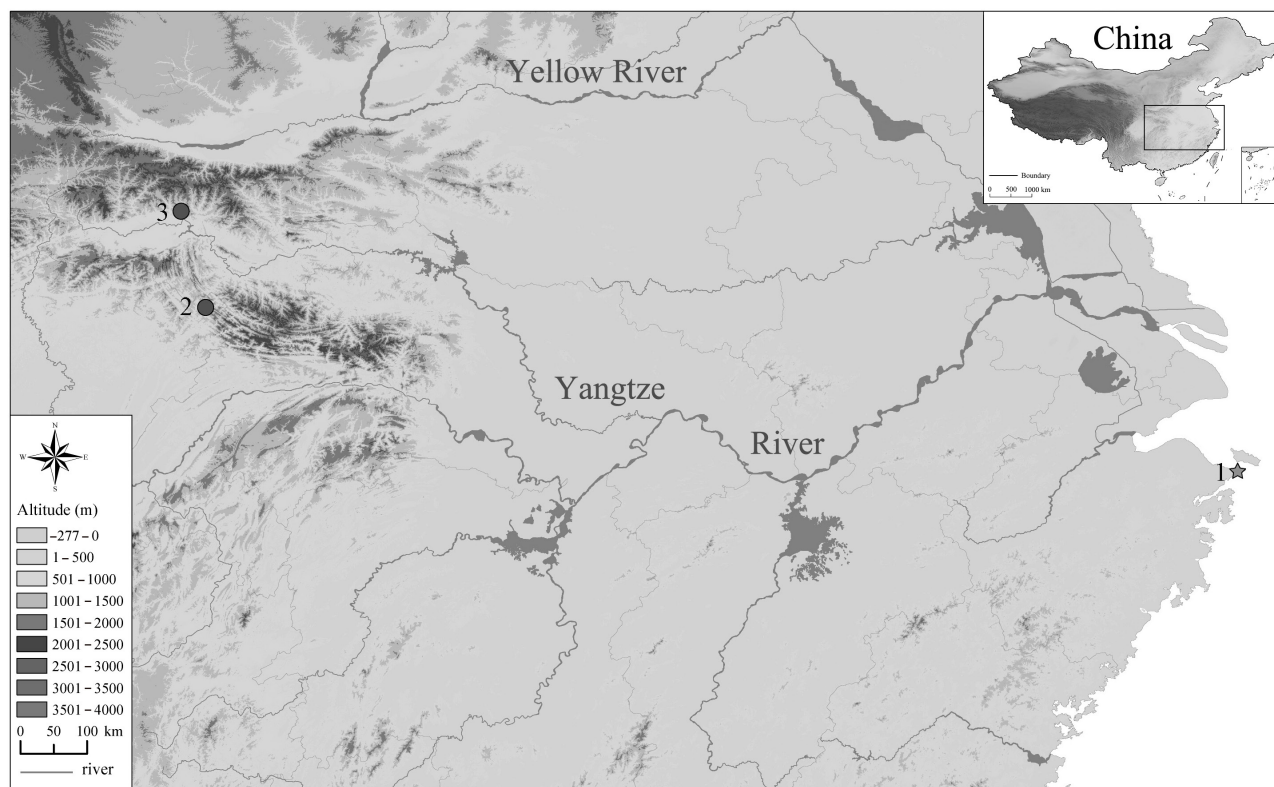
on morphology (e.g. skin smoothness, colour spots, supratympanic fold and canthus rostralis) between the population from the type locality, Hua'e mountain of Sichuan Province of China, and other populations, such as, populations from Yangxian County of Shaanxi Province (Hu *et al.*, 1966), Shangcheng County of Henan Province (Chen *et al.*, 2003), and Beilun District of Ningbo City of Zhejiang Province (Fei *et al.*, 1999, 2012). These work indicated that there may be cryptic species in *M. mixtura* complex. However, to now, there has been no molecular phylogenetic examination in this group, which was proved to be effective in evaluating species diversity or species boundary in Microhylidae (e.g. Howlader *et al.*, 2015; Khatiwada *et al.*, 2017; Matsui, 2011; Matsui *et al.*, 2011).

From 1997 to 2017, we collected 41 adult specimens of the genus *Microhyla* from Beilun District, Ningbo City, Zhejiang Province of China, which were initially identified as *M. mixtura* (Huang *et al.*, 1990; Fei *et al.*, 1999, 2012). Our morphological comparisons and phylogenetic analyses consistently suggested that these specimens were distinctly different from its congeners. Therefore, we described it as a new species in this study.

## 2. Materials and Methods

**2.1. Sampling** During April 1997, March to April 1998, and April 2017, a total of 41 adult specimens (list in Table S1) initially classified into *M. mixtura* were collected from Chaiqiao Town (29.86667° N, 121.55000° E, 120 m a.s.l.), Beilun District, Ningbo City, Zhejiang Province of China (Figure 1; here after we called Beilun population). For comparisons, ten adult specimens of *M. mixtura* from Hua'e mountain of Sichuan Province, the type locality of the species (here after we called Hua'e mountain population), and eight adult specimens of it from Yangxian County of Shaanxi Province (here after we called Yangxian population; list in Table S1; Figure 1). All specimens were fixed in 10% buffered formalin for one day, and then transferred to 70% ethanol. Tissues samples were preserved separately in 95% ethanol prior to fixation. Specimens collected in this work were deposited in Chengdu Institute of Biology (CIB), Chinese Academy of Sciences (CAS), Chengdu, China.

**2.2. Molecular phylogenetics** For molecular work, five samples from Beilun population, eight samples from



**Figure 1** Sampling localities in this study. 1, the type locality of *Microhyla beilunensis* sp. nov., Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China; 2, the type locality of *M. mixtura*, Hua'e Mountain, Wanyuan City, Sichuan Province, China; 3, another locality of *M. mixtura*, Huayang Town, Yangxian County, Shaanxi Province, China.

Hua'e mountain population and eight samples from Yangxian population of *M. mixtura* were used (list see Table 1).

Total genomic DNA was extracted from the muscles using a DNA extraction kit (Tiangen Biotech, Beijing).

For molecular analyses, DNA fragments of mitochondrial

**Table 1** Vouchers, localities and GenBank accession numbers for samples used in the molecular analyses.

ID	Species	Voucher	Locality	GenBank Accession No.		
				12S	16S	CO1
1	<i>Microhyla mixtura</i>	CIBZMH2017061201	Yangxian, Hanzhong, Shaanxi, China	MH234526	MH234532	MH234549
2	<i>Microhyla mixtura</i>	CIBZMH2017061202	Yangxian, Hanzhong, Shaanxi, China	MH234527	MH234533	MH234550
3	<i>Microhyla mixtura</i>	CIBZMH2017061203	Yangxian, Hanzhong, Shaanxi, China	MH234528	MH234534	MH234551
4	<i>Microhyla mixtura</i>	CIB20170526001	Hua'e mountain, Wanyuan, Sichuan, China	MH234529	MH234540	MH234557
5	<i>Microhyla mixtura</i>	CIB20170526002	Hua'e mountain, Wanyuan, Sichuan, China	MH234530	MH234541	MH234558
6	<i>Microhyla mixtura</i>	CIB20170526003	Hua'e mountain, Wanyuan, Sichuan, China	MH234531	MH234542	MH234559
7	<i>Microhyla beilunensis</i> sp. nov.	CIBBL002	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234521	MH234535	MH234543
8	<i>Microhyla beilunensis</i> sp. nov.	CIBBL003	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234522	MH234536	MH234544
9	<i>Microhyla beilunensis</i> sp. nov.	CIBBL004	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234523	MH234537	MH234545
10	<i>Microhyla beilunensis</i> sp. nov.	CIBBL005	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234524	MH234538	MH234546
11	<i>Microhyla beilunensis</i> sp. nov.	CIBBL006	Chaiqiao, Beilun, Ningbo, Zhejiang, China	MH234525	MH234539	MH234547
12	<i>Microhyla okinavensis</i>	KUHE12840	Amamioshima, Japan	*AB201173	*AB201184	-
13	<i>Microhyla mukhlesuri</i>	Morn-Bd12	Raozan, Chittagong, Bangladesh	-	*AB543609	-
14	<i>Microhyla mymensinghensis</i>	Morn-Bd9	Golapganj, Sylhet, Bangladesh	-	*AB543606	-
15	<i>Microhyla fissipes</i>	KUHE32943	Huangshan, Anhui, China	*AB201174	*AB201185	-
16	<i>Microhyla borneensis</i>	KUHE53938	Serapi, Sarawak, Malaysia	*AB634605	*AB634663	-
17	<i>Microhyla malang</i>	KUHE53018	Serapi, Sarawak, Malaysia	*AB598295	*AB598319	-
18	<i>Microhyla orientalis</i>	KUHE55073	Wongaya Gede, Bali, Indonesia	*AB781458	*AB781469	-
19	<i>Microhyla mantheyi</i>	KUHE52556	Temerloh, Pahang, Malaysia	*AB598310	*AB598334	-
20	<i>Microhyla achatina</i>	MDK24	Gede Pangrango, Java, Indonesia	*AB634599	*AB634657	-
21	<i>Microhyla heymonsi</i>	KUHEUN(K1845)	Kanchanaburi, Thailand	*AB201179	*AB201190	-
22	<i>Microhyla beidmorei</i>	MZBAmp15270	Paramasan, Kalimantan, Indonesia	*AB634603	*AB634661	-
23	<i>Microhyla fowleri</i>	KUHE21992	Mae Yom, Phrae, Thailand	*AB634609	*AB634667	-
24	<i>Microhyla pulchra</i>	KUHE35119	Phu Luan, Loei, Thailand	*AB201180	*AB201191	-
25	<i>Microhyla sholigari</i>	ATREE_MISH_1	-	*KT600667	*KT600674	-
26	<i>Microhyla laterite</i>	BNHS5967	Manipal, Udipi District, Karnataka, India	*KT600664	*KT600671	-
27	<i>Microhyla superciliaris</i>	KUHE53371	Kenaboi, Negeri Sembilan, Malaysia	*AB634625	*AB634683	-
28	<i>Microhyla butleri</i>	KUHE40591	A Luoi, A Roang, Vietnam	*AB634606	*AB634664	-
29	<i>Microhyla palmipes</i>	MZB Amp16255	Bedegul, Bali, Indonesia	*AB634612	*AB634670	-
30	<i>Microhyla ornata</i>	ZSIK-A9119	Karnataka, India	*AB201177	*AB201188	-
31	<i>Microhyla taratiensis</i>	JRK201525	Jamun Khadi, Jhapa District, Nepal	-	*KY655952	-
32	<i>Microhyla nilphamariensis</i>	JRK201522	Jamun Khadi, Nepal	-	*KY655947	-
33	<i>Microhyla rubra</i>	released (toe tip)	Karnataka, India	*AB201181	*AB201192	-
34	<i>Microhyla annectens</i>	KUHE53373	Genting, Selangor, Malaysia	*AB634600	*AB634658	-
35	<i>Microhyla marmorata</i>	KUHE32455	Xamneua, Houapan, Laos	*AB634610	*AB634668	-
36	<i>Microhyla perparva</i>	KUHE53675	Mulu, Sarawak, Malaysia	*AB634615	*AB634673	-
37	<i>Microhyla petrigena</i>	BORN22412	Maliau Basin, Sabah, Malaysia	*AB634616	*AB634674	-
38	<i>Microhyla nanapollexa</i>	AMNH:Herp:163686	Tra My District, Quang Nam, Vietnam	-	*KM509164	-
39	<i>Chaperina fusca</i>	BORN8479	Crocker, Sabah, Malaysia	*AB781451	*AB781466	-
40	<i>Microhyla mixtura</i>	CIBZMH2017061204	Yangxian, Hanzhong, Shaanxi, China	-	-	MH234552
41	<i>Microhyla mixtura</i>	CIBZMH2017061205	Yangxian, Hanzhong, Shaanxi, China	-	-	MH234553
42	<i>Microhyla mixtura</i>	CIBZMH2017061206	Yangxian, Hanzhong, Shaanxi, China	-	-	MH234554
43	<i>Microhyla mixtura</i>	CIBZMH2017061207	Yangxian, Hanzhong, Shaanxi, China	-	-	MH234555
44	<i>Microhyla mixtura</i>	CIBZMH2017061208	Yangxian, Hanzhong, Shaanxi, China	-	-	MH234556
45	<i>Microhyla mixtura</i>	CIB20170526008	Hua'e mountain, Wanyuan, Sichuan, China	-	-	MH234560
46	<i>Microhyla mixtura</i>	CIB20170526009	Hua'e mountain, Wanyuan, Sichuan, China	-	-	MH234561
47	<i>Microhyla mixtura</i>	CIB20170526010	Hua'e mountain, Wanyuan, Sichuan, China	-	-	MH234562
48	<i>Microhyla mixtura</i>	CIB20170526011	Hua'e mountain, Wanyuan, Sichuan, China	-	-	MH234563
49	<i>Microhyla mixtura</i>	CIB20170526012	Hua'e mountain, Wanyuan, Sichuan, China	-	-	MH234564
50	<i>Microhyla butleri</i>	CIBSZ20150408	Shenzhen, Guangdong, China	-	-	*NC_030049

(Continued Table 1)

ID	Species	Voucher	Locality	GenBank Accession No.		
				12S	16S	CO1
51	<i>Microhyla butleri</i>	-	Bantai valley, Kuala Lumpur, Malaysia	-	-	*KT285802
52	<i>Microhyla butleri</i>	MVZ223728	Vinh Phu Province, Vietnam	-	-	*EF396054
53	<i>Microhyla heymonsii</i>	-	-	-	-	*NC_006406
54	<i>Microhyla heymonsii</i>	-	-	-	-	*AY458596
55	<i>Microhyla heymonsii</i>	-	-	-	-	*EF396055
56	<i>Microhyla okinavensis</i>	-	Ishigaki Isl., Okinawa, Japan	-	-	*NC_010233
57	<i>Microhyla okinavensis</i>	IABHU5263	Ishigaki Isl., Okinawa, Japan	-	-	*AB303950
58	<i>Microhyla ornata</i>	-	-	-	-	*NC_009422
59	<i>Microhyla ornata</i>	-	-	-	-	*DQ512876
60	<i>Microhyla pulchra</i>	-	Yingping mountain, Guangdong, China	-	-	*NC_024547
61	<i>Microhyla pulchra</i>	-	Yingping mountain, Guangdong, China	-	-	*KF798195
62	<i>Microhyla pulchra</i>	MVZ223797	Vinh Phu, Vietnam	-	-	*EF396056

\* denotes the gene sequences downloaded from GenBank.

12S rRNA, 16S rRNA, and CO1 genes were amplified and sequenced for 11 samples, 11 samples and 21 samples, respectively (see Table 1). Primers were presented in Table 2. PCR amplifications were performed as following conditions: an initial denaturing step at 95 °C for 4 min; followed by 35 cycles of denaturing at 94 °C for 35 s, annealing at 46-52 °C for 30s, then extending at 72 °C for 1 min, and the final extending step of 72 °C for 10 min. PCR products were purified using a Qiagen PCR purification kit, and sequenced on an ABI3500 automated sequencer using the front/back primers same to PCR. The newly sequences were deposited in GenBank (GenBank accession numbers were shown in Table 1).

Sequences were aligned using the clustal walgorithm in MEGA v. 6.0 (Tamura *et al.*, 2013) with default parameters, and then checked by eyes for accuracy. For phylogenetic analyses, we downloaded the compiled available sequence data for the related species from GenBank (GenBank accession numbers see Table 1). Concatenated sequence data of 12S (387 bp) and 16S (540 bp) of 29 *Microhyla* species were used for phylogenetic reconstructions. According to Matsui *et al.* (2011), one *Chaperina fusca* sample was chosen as outgroup.

Phylogenetic relationships were reconstructed by Maximum likelihood (ML) and Bayesian Inference (BI) methods, implemented in RAxMLHPC v. 8.1.20 (Stamatakis *et al.*, 2006) and MrBayes v. 3.2 (Ronquist *et al.*, 2012), respectively. Before that, the best-fit substitution model was selected by jModeltest v. 2.1.4 (Darriba *et al.*, 2012) under the corrected Akaike Information Criterion (AICc; Hurvich and Tsai, 1989), and as results, GTR+I+G model and GTR+I+G+X model were selected for the BI and ML phylogenetic analyses, respectively. Non-parametric bootstrapping with heuristic

searches of 1000 replicates was used to assess confidences of branches in ML trees. In BI, we initiated two dependent runs each with four simultaneous Monte Carlo Markov chains (MCMC) for 20 million generations with sampling every 1000 generations and discarded the first 25% of generations as burn-in after the convergence of chains was confirmed. The final majority tree and posterior probabilities (pp) were achieved from the remaining trees. In addition, to further visualize the degree of genetic splits among the new taxon and its closely-related species, we reconstructed a phylogenetic network based on the CO1 gene sequences dataset using maximum parsimony method in SplitsTree v. 4.11.3 (Huson and Bryant, 2006). The supports of Splittree lineages were evaluated by 1000 bootstrap replicates. Finally, we calculated pairwise uncorrected *p*-distance between the newly-found taxon and its congeners by the combination of 12S rRNA and 16S rRNA gene sequences in MEGA.

**2.3. Morphological comparisons** We compared morphological characters of the Beilun population with other *Microhyla* species. Morphological data for comparisons were obtained from the available literatures (Andersson, 1942; Bain and Nguyen, 2004; Blyth, 1856; Boulenger, 1884, 1897, 1990; Das *et al.*, 2007; Duméril and Bibron, 1841; Dutta and Ray, 2000; Fernando and Siriwardhane, 1996; Hallowell, 1861; Hasan *et al.*, 2014; Howlader *et al.*, 2015; Inger, 1989; Inger and Frogner, 1979; Jerdon, 1853; Khatiwada *et al.*, 2017; Hu *et al.*, 1966; Matsui, 2011; Matsui *et al.*, 2013; Parker, 1928, 1934; Parker and Osman-Hill, 1948; Pillai, 1977; Poyarkov *et al.*, 2014; Schenkel, 1901; Seshadri *et al.*, 2016; Smith, 1923; Stejneger, 1901; Tschudi, 1838; Vogt, 1911; Wijayathilaka *et al.*, 2016).

For morphometric analyses, 41 specimens of Beilun



**Table 2** Primers used in this study.

Gene	Primer	Sequence	Approximate product size (bps)	Annealing temperature (°C)	Reference
12S	P1	GCT TCA AAC TGG GAT TAG ATA CCC CAC TAT	385	52	Kocher <i>et al.</i> , 1989
	P2	TGA CTG CAG AGG GTG ACG GGC GGT GTG T			
16S	P7	CGC CTG TTT ATC AAA AAC AT	550	49	Simons <i>et al.</i> , 1994
	P8	CCG GTC TGA ACT CAG ATC ACG T			
COI	chmf4	TYT CWA CWA AYC AYA AAG AYA TCG G	590	46	Che <i>et al.</i> , 2012
	chmr4	ACY TCR GGR TGR CCR AAR AAT CA			

population, and ten adult specimens of Hua'e mountain population were measured. Morphological measurements were taken with a digital caliper to the nearest 0.01 mm. The terminology and methods follows Fei *et al.* (2005) and Watters *et al.* (2016). Totally 16 characters were measured: snout-vent length (SVL, distance from the tip of the snout to the posterior edge of the vent), head length (HL, distance from the tip of the snout to the articulation of jaw), head width (HW, greatest width between the left and right articulations of jaw), snout length (SL, distance from the tip of the snout to the anterior corner of the eye), eye diameter (ED, distance from the anterior corner to the posterior corner of the eye), internasal distance (IND, minimum distance between the inner margins of the external nares), interorbital distance (IOD, minimum distance between the inner edges of the upper eyelids), upper eyelid width (UEW, greatest width of the upper eyelid margins measured perpendicular to the anterior-posterior axis), lower arm and hand length (LAHL, distance from the elbow to the distal end of the Finger IV), lower arm width (LAW, maximum width of the lower arm), hand length (HAL, distance from the posterior end of the inner metacarpal tubercle to the distal tip of Finger IV), hindlimb length (HLL, maximum length from the vent to the distal tip of the Toe IV), tibia length (TL, distance from the outer surface of the flexed knee to the tibiotarsal articulation), tibia width (TW, maximum width of the tibia), length of tarsus and foot (TFL, distance from the tibiotarsal articulation to the distal end of the Toe IV), length of foot (FL, distance from the posterior end of the inner metatarsal tubercle to the distal tip of the Toe IV).

Principal component analysis (PCA) of size-corrected valuables and simple bivariate scatterplots were used to explore and reflect the morphometric differences between the Beilun population and Hua'e mountain population. Independent-Sample *T* test was applied to further explore the morphometric differences between these two populations. Independent-Sample *T* test was also conducted to test the morphometric differences between the males and the females of the new species. The statistical analyses were performed using SPSS v. 21.0

(SPSS, Inc., Chicago, IL, USA), and differences were considered significant at  $P < 0.05$ .

### 3. Results

**3.1. Phylogenetic analyses** The aligned dataset of the mitochondrial 12S and 16S genes contained 927 bp, in which 608 sites were variable, and 304 were parsimony sites. BI and ML analyses resulted in a consistent topology (Figure 2A). Specimens of Beilun population were clustered into one clade, which was sister to the *M. mixtura* and *M. okinavansis* clades. SplitTree analyses based on COI gene sequences also showed that the Beilun population formed an independent lineage, which was distinctly separated from its closely-related species (Figure 2B). In addition, the estimated genetic distance based on 12S and 16S gene dataset (Table 3) between the Beilun population and *M. mixtura* populations was 2.6%, being higher than that between *M. mukhlesuri* and *M. fissipes* (2.4%) and equal to that between *M. malang* and *M. borneensis* (2.6%).

**3.2. Morphological analyses** According to the available morphological data concerning *Microhyla* taxa, the Beilun population differs from *M. annamensis*, *M. annectens*, *M. berdmorei*, *M. darevskii*, *M. laterite*, *M. malang*, *M. mantheyi*, *M. marmorata*, *M. nanapollexa*, *M. perparva*, *M. petrigena*, *M. pulchella*, *M. pulverata*, *M. sholigari* by presence of basal rudimentary webbing (versus well developed toe webbing reaching disks or reaching distal subarticular tubercles); differentiated from *M. arboricola*, *M. achatina*, *M. butleri*, *M. fusca*, *M. heymonsi*, *M. minuta*, *M. orientalis*, *M. pineticola*, *M. pulchella*, and *M. karunaratnei* by having disks only on the toes (versus both fingers and toes disks present); distinguish from *M. fissipes*, *M. mihintalei*, *M. mukhlesuri*, *M. mymensinghensis*, *M. niphamariensis*, *M. pulchra*, *M. picta*, *M. rubra*, *M. ornata*, *M. inornata*, *M. taraiensis* by presenting toe disks (versus both finger and toe disks entirely absent); distinct from *M. zeylanica*, *M. chakrapanii*, *M. maculifera*, *M. palmipes*, *M. superciliaris*

**Table 3** Uncorrected *p*-distance between *Microhyla* species based on 12S and 16S gene sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. <i>Microhyla beilunensis</i> sp. nov.																
2. <i>M. Mixtura</i> (Hua'e mountain, Sichuan)	0.026															
3. <i>M. Mixtura</i> (Yangxian County, Shaanxi)	0.026	0.005														
4. <i>M. okinavensis</i>	0.031	0.037	0.037													
5. <i>M. nymensinghensis</i>	0.044	0.050	0.051	0.065												
6. <i>M. mukhtesuri</i>	0.042	0.050	0.051	0.061	0.033											
7. <i>M. fissipes</i>	0.043	0.042	0.043	0.052	0.029	0.024										
8. <i>M. malang</i>	0.070	0.069	0.071	0.077	0.073	0.067	0.071									
9. <i>M. borneensis</i>	0.072	0.072	0.071	0.080	0.075	0.079	0.075	0.026								
10. <i>M. orientalis</i>	0.065	0.071	0.070	0.072	0.077	0.075	0.070	0.049	0.049							
11. <i>M. mantheyi</i>	0.063	0.065	0.064	0.068	0.073	0.075	0.066	0.064	0.051	0.051						
12. <i>M. achatina</i>	0.074	0.075	0.075	0.075	0.078	0.078	0.073	0.070	0.068	0.066	0.057					
13. <i>M. heymonsii</i>	0.059	0.062	0.063	0.067	0.063	0.059	0.060	0.062	0.063	0.065	0.057	0.076				
14. <i>M. berdmorei</i>	0.084	0.080	0.079	0.086	0.100	0.096	0.086	0.091	0.086	0.094	0.081	0.076	0.079			
15. <i>M. fowleri</i>	0.076	0.077	0.077	0.080	0.098	0.098	0.082	0.086	0.079	0.083	0.071	0.067	0.072	0.018		
16. <i>M. pulchra</i>	0.070	0.077	0.077	0.074	0.096	0.090	0.083	0.096	0.098	0.094	0.084	0.082	0.073	0.063	0.058	
17. <i>Chaperina fusca</i>	0.156	0.160	0.161	0.161	0.167	0.163	0.164	0.168	0.165	0.165	0.157	0.164	0.162	0.169	0.166	0.163

by presence of dorsal median longitudinal grooves on the toe disks (versus absent of longitudinal grooves on toe disks).

The Beilun population could be identified from Hua'e mountain population in the following characters: 1) apart from the stripes, bar-shaped and oval-shaped patterns, the rounded spots present on the dorsum of body and legs (versus absent in the latter); 2) the outer metacarpal tubercles prominently larger than the inner one (versus the outer metacarpal tubercle slightly larger than the inner one in the latter).

Meanwhile, the results of morphometric analyses indicated that the Beilun population could be well separated from Hua'e mountain population. PCA extracted four principal component axes with eigenvalues greater than one, and of these, the first two component axes accounted for 53.34% of the variation (Table 4). The first two principal component axes could separate the Beilun population from Hua'e mountain population (Figure 3) mainly based on limb and head characteristics, namely, TFL, FL, TL, HLL, LAHL, SL, IOD, HL, UEW and LAW (Table 4). Independent-Sample *T* test showed that in male, there are significant differences on 11 morphometric characters between Beilun population and Hua'e mountain population: the ratios of HW, IND, UEW and LAW to SVL of Beilun population were significantly larger than those of Hua'e mountain population ( $P < 0.01$ ), and the ratios of SL, IOD, LAHL, HLL, TL, TFL and FL to SVL of Beilun population were significantly less than those of Hua'e mountain population ( $P < 0.05$ , Table S2).

**3.3. Taxonomic conclusion** The results of morphological and molecular phylogenetic analyses supported that Beilun population was distinctly differed from its congeners. Thus, we described it as a new species here:

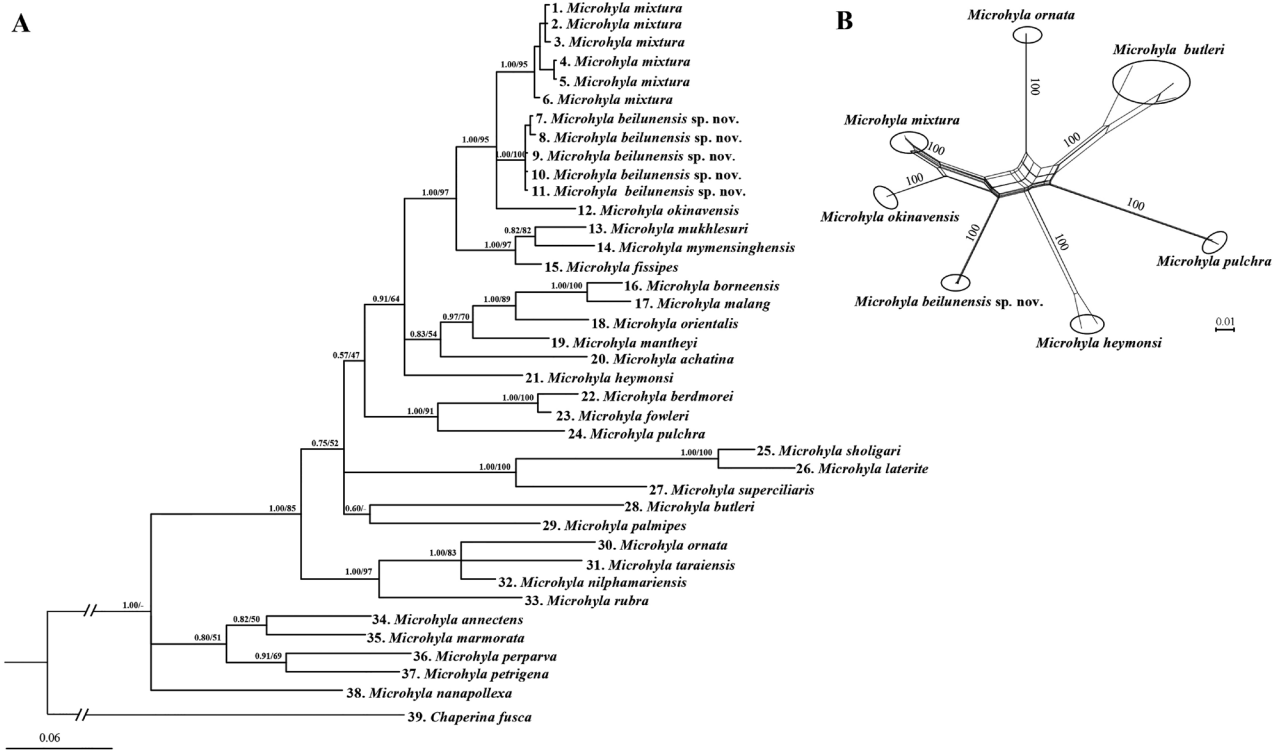
***Microhyla beilunensis* sp. nov.**

*Microhyla mixtura*: Fei, Ye, Xie and Cai. 1999. Chin Zool Res, Beijing (Beilun, Zhejiang): 239–240.

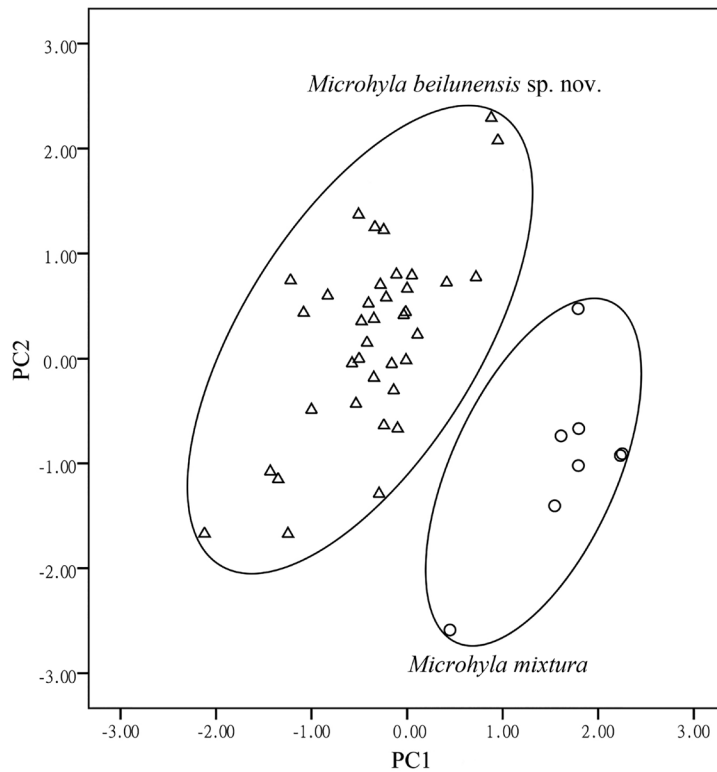
**Holotype**: CIBA980059, adult male (Figure 4A, C, E, G, I, K), SVL 22.09 mm, from Chaiqiao Town (29.86667 °N, 121.55000 °E, 120 m a.s.l), Beilun District, Ningbo City, Zhejiang Province, China (Figure 1), collected by Liang Fei on 25 April 1998.

**Allotype**: CIBA980062, adult female (Figure 5), SVL 28.02 mm, collected on the same day and the same location by Liang Fei.

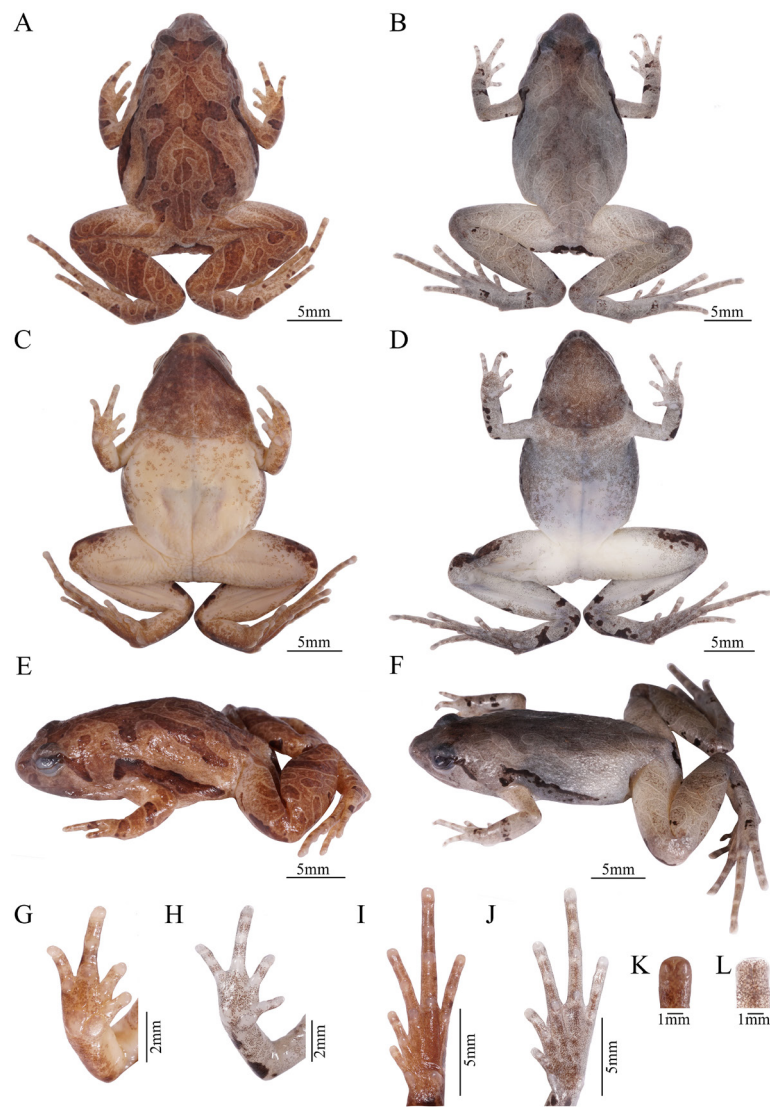
**Paratype**: 36 adult males (CIBA97001–CIB A97023, CIBA98060, CIBA98063–CIBA98067) and three adult



**Figure 2** Phylogenetic relationships within the genus *Microhyla*. A, the Bayesian inference tree for 29 *Microhyla* species reconstructed based on 12S rRNA and 16S rRNA gene sequences. Bayesian posterior probabilities/maximum likelihood bootstrap supports were noted near each node, the symbol“-” indicates value below 50. B, phylogenetic network constructed by SplitTree for seven *Microhyla* species based on COI gene sequences.



**Figure 3** Plots of the first principal component (PC1) versus the second (PC2) for the males of *Microhyla beilunensis* sp. nov. and toptype *M. mixtura*.



**Figure 4** Morphological comparisons between *Microhyla beilunensis* sp. nov. and *M. mixtura*. *Microhyla beilunensis* sp. nov. (holotype, male): dorsal view (A), ventral view (C), lateral view (E), ventral view of hand (G), ventral view of foot (I), dorsal median longitudinal grooves of Toe IV (K). *M. mixtura* (topotype, male): dorsal view (B), ventral view (D), lateral view (F), ventral view of hand (H), ventral view of foot (J), dorsal median longitudinal grooves of Toe IV (L).

females (CIBA97024, CIBA97025, CIBA980061) were collected from the type locality in April 1997, March to April in 1998 by Liang Fei, and seven adult males (CIBBL001–CIBBL007) were collected in April in 2017 by Yufan Wang.

These specimens are all deposited in Chengdu Institute of Biology, Chinese Academy of Sciences (CIB, CAS), Chengdu, China.

**Etymology:** This specific name *beilunensis* is a Latinized toponymic adjective that refers to Beilun District, Ningbo City, Zhejiang Province of China, where the new species was collected. For the common name, we suggest Beilun Pygmy frog (English) and Bei lun ji wa (Chinese).

**Diagnosis:** The new species is allocated to the genus *Microhyla*, because it is consistent with the generic diagnostic characters: relatively small body size; maxillary and vomerine teeth absent; vomer divided into two parts and disappearing at the posterior edge of the choana; tongue posteriorly rounded; skin smooth or with tubercles; tympanum hidden or indistinct; 1–2 row of horizontal skin ridges on the palate; fingers without webbing; toes webbed or free of webbing; 2 or 3 metacarpal tubercles; the absence of supernumerary tubercles below the base of fingers and toes; the absence of skin ridge or skin projection between the subarticular tubercles of toes III and IV (Parker, 1934; Fei *et al.*, 2005, 2009).



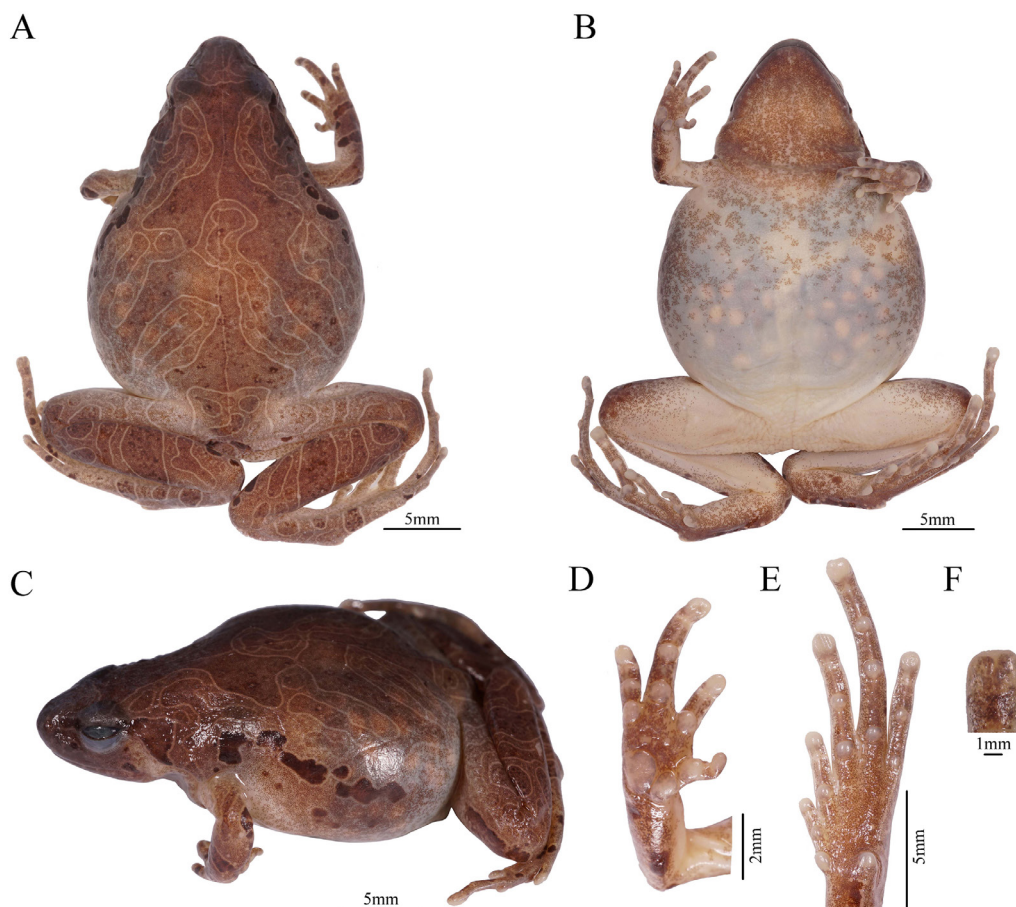
**Table 4** Variable loadings for principal components with Eigenvalue greater than 1, from morphometric characters corrected by SVL. All measurements were given in millimeter (mm).

Morphological features	PC1	PC2	PC3	PC4
SVL	-0.29	-0.61	0.20	0.31
HL	0.10	0.81	-0.15	0.06
HW	-0.13	0.75	0.23	0.22
SL	0.64	0.29	0.09	-0.25
IND	-0.29	0.63	0.05	0.15
IOD	0.61	0.38	-0.37	-0.07
UEW	-0.17	0.81	-0.08	0.20
ED	-0.15	0.23	0.23	-0.84
LAHL	0.81	0.12	0.00	-0.11
LAW	-0.28	0.68	0.25	0.03
HAL	0.36	0.19	-0.39	0.01
HLL	0.86	-0.06	0.16	-0.01
TL	0.87	-0.04	0.13	0.18
TW	0.21	0.14	0.79	0.07
TFL	0.92	-0.21	0.08	0.06
FL	0.88	0.05	0.02	0.17
Eigenvalues	5.04	3.50	1.21	1.06
Percentage of variance	31.48	21.86	7.57	6.62

The new species could be identified from its congeners except *M. mixtura* by the several characters: (1) rudimentary webs on toe base; (2) absence of disks and dorsal median longitudinal grooves on finger tips; (3) presence of disks and dorsal median longitudinal grooves on toe tips. As well, the new species could be identified from Hua'e mountain population in the following characters: 1) apart from the stripes, bar-shaped and oval-shaped patterns, the rounded spots present on the dorsum of body and legs; 2) the outer metacarpal tubercles prominently larger than the inner one.

In male, between the new species and *M. mixtura*, there is significant differences on eleven morphometric characters: 1) the ratios of HW, IND, UEW and LAW to SVL of the new species were significantly larger than those of *M. mixtura* ( $P < 0.01$ ); 2) the ratios of SL, IOD, LAHL, HLL, TL, TFL and FL to SVL of the new species were significantly less than those of *M. mixtura* ( $P < 0.05$ ).

**Description of holotype:** Small body size (SVL 22.09 mm). Head length 75% head width. Snout short, rounded,



**Figure 5** Allotype of *Microhyla beilunensis* sp. nov. (CIBA980062, adult female, SVL 28.02 mm). A, dorsal view; B, ventral view; C, lateral view; D, ventral view of hand; E, ventral view of foot; F, dorsal median longitudinal groove of Toe IV.





**Figure 6** Variation in *Microhyla beilunensis* sp. nov. from Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China. A and B, dorsal and ventral views of the holotype in life, respectively; C and D, dorsal and ventral views of the allotype in life, respectively; E, mating couples; F, breeding site.



and projecting beyond the lower jaw. Nostril rounded, distinct, and obviously closer to the tip of the snout than the eye. Interorbital distance (2.46 mm) greater than internasal distance (2.10 mm) and upper eyelid width (1.60 mm), but smaller than eye diameter (3.09 mm). Canthus rostralis and supratympanic fold distinct, tympanum hidden. Tongue posteriorly oval. Maxillary and vomerine edentate.

Forelimbs relatively short and thin. Lower arm (14% of SVL) shorter than hand (24% of SVL). Fingers short, distally rounded, not webbed, disks and dorsal longitudinal grooves absent, the relative finger lengths: III > IV > II > I. Subarticular tubercles roughly circle-shaped and distinct. Supernumerary tubercles below the base of finger absent. Inner and outer metacarpal tubercles prominent, oval-shaped, the inner one distinctly larger than the outer one, which close to each other (Figure 4 G). Nupital pads and spines absent.

Hind limb length 170% of SVL, tibia length 55% of SVL, length of tarsal and foot 76% of SVL, and foot length 53% of SVL. Tibiotarsal articulation reaching the eyes or the posterior margin of the eyes when hindlimb extend along the body side. Heels overlapped when hind limbs folded at right angles. Toes distally rounded, and equipped with disks and longitudinal grooves except for the toe I, webbing poorly developed at base of toes, relative toe lengths: IV > III > V > II > I. Inner metatarsal tubercle long and thin, while outer metatarsal tubercle relatively small and rounded, they widely separated from each other (Figure 4I). Subarticular tubercles

approximately circle-like and markedly prominent. Supernumerary tubercles below the base of toes absent.

Dorsal skin surface roughish with dense tiny tubercles. Ventral surface of posterior part of body, cloacal region, thigh granules. Throat, chest, and ventral part of thigh and tibia smooth.

**Color in life:** Dorsal body brownish or gray-brownish, the presence of dark brown markings with light brown margins (Figure 6A, C, E). A thick dark brown butterfly marking between the eyes and on the dorsal body with three pairs of symmetrical figures or streaks or projections, the anterior pair of figures shorter and extending laterally to the middle of upper eyelid; the middle pair relatively long and extending laterally; posterior pair of streaks running obliquely from above the shoulder to the hip, these streaks in connection with each another or not. Dorsum of posterior part of body and legs brownish scattered with dense dark brown circle-like spots; rounded, bar-shaped pattern and streaks with light margins. Supratympanic fold dark brown. Spots on body sides contiguous or not. Limbs indefinitely barred with dark brown. One back brown patches on anterior surface of thigh. Vent light brown with a large black patch. No black and yellowish streaks on the flanks, throat dark purple, chest and belly white mottled with purple (Figure 6B, D).

**Variation:** The morphometric variation of 37 adult males and 4 adult females (listed in Table S1). Basic statistics for morphological measurements was presented

**Table 5** Measurements of *Microhyla beilunensis* sp. nov. and *M. mixtura*. All measurements were given in millimeter (mm).

	<i>Microhyla beilunensis</i> sp. nov.				<i>M. mixtura</i>			
	Female (n = 4)		Male (n = 37)		Female (n = 2)		Male (n = 8)	
	Mean ± SD	Range	Mean ± SD	Range	Mean	Range	Mean ± SD	Range
SVL	27.58 ± 0.83	26.39–28.25	21.86 ± 1.16	19.08–23.73	25.52	24.84–26.20	22.16 ± 1.94	18.77–25.19
HL	7.40 ± 0.50	6.83–7.97	6.36 ± 0.36	5.68–7.15	7.26	7.08–7.44	6.20 ± 0.29	5.77–6.59
HW	8.37 ± 0.63	7.47–8.93	7.72 ± 0.51	7.00–9.35	7.39	7.18–7.60	7.32 ± 0.63	6.27–8.15
SL	3.13 ± 0.23	2.81–3.36	2.71 ± 0.18	2.28–3.03	3.13	2.83–3.42	2.90 ± 0.14	2.73–3.08
IND	2.30 ± 0.06	2.25–2.35	2.13 ± 0.23	1.86–3.04	1.99	1.82–2.16	1.92 ± 0.17	1.75–2.22
IOD	2.83 ± 0.21	2.61–3.06	2.53 ± 0.26	2.07–3.00	2.95	2.92–2.99	2.81 ± 0.23	2.47–3.12
UEW	1.94 ± 0.14	1.76–2.11	1.68 ± 0.14	1.34–1.90	1.74	1.74–1.75	1.51 ± 0.10	1.37–1.69
ED	3.10 ± 0.16	2.94–3.31	2.79 ± 0.27	1.98–3.27	3.15	3.08–3.23	2.72 ± 0.14	2.49–2.89
LAHL	9.99 ± 0.37	9.45–10.29	8.72 ± 0.35	7.77–9.39	9.35	8.90–9.80	9.60 ± 0.86	7.85–10.47
LAW	1.43 ± 0.03	1.39–1.45	1.42 ± 0.11	1.08–1.67	1.32	1.19–1.45	1.28 ± 0.13	1.07–1.43
HAL	6.14 ± 0.39	5.77–6.69	5.43 ± 0.81	4.76–9.99	6.02	5.84–6.19	5.79 ± 0.54	4.65–6.52
HLL	41.18 ± 1.52	39.80–42.63	35.10 ± 1.87	30.16–37.85	43.03	40.69–45.38	38.93 ± 3.11	32.98–42.74
TL	13.80 ± 0.78	12.85–14.51	11.70 ± 0.69	9.02–12.71	14.00	13.13–14.86	13.12 ± 0.99	11.10–14.38
TW	3.87 ± 0.13	3.72–4.03	3.41 ± 0.26	2.96–4.02	3.36	3.17–3.54	3.44 ± 0.39	2.64–3.82
TFL	19.55 ± 0.99	18.52–20.53	13.26 ± 0.90	13.89–17.66	19.67	18.80–20.53	18.46 ± 1.46	15.40–20.29
FL	13.15 ± 0.25	12.85–13.37	11.46 ± 0.61	9.85–12.48	13.65	13.39–13.91	12.76 ± 0.92	10.94–14.18

in Table 5. The butterfly dorsal marking interrupted behind head in some individuals. Behind the butterfly marking, bar-shaped and rounded patterns presented in most individuals; but in some specimens, there one or two stripes parallel with the posterior part of the butterfly marking. Also, the number of spots on body variation, such as, spots on ventral of forelimbs 0–6, those on posterior part of thigh 0–4, those on anterior surface of shank 1–9, and those on posterior surface of shank 2–8. Two patterns near the vent continuous or discontinuous.

**Sexual dimorphism:** The snout-vent length of females significantly larger than that of males ( $t_{39} = -9.58$ ,  $P < 0.001$ ). Besides, the ratios of HL, HW, SL, IND, ED, LAHL, LAW, HLL, TL, TW, TFL and FL to SVL of females were significantly greater than those of males ( $P < 0.05$ ; Table S3). Adult males with a single external subgular vocal sac, dark purple throat, occipital furrow remarkably prominent, pink linea musculina present on dorsum and belly of body. Nupital pads and spines absent. The gravid females containing brownish oocytes in oviduct which clearly visible through the belly skin.

**Distribution and habits:** *Microhyla beilunensis* sp. nov. is now known only from the type locality, Beilun District, Ningbo City, Zhejiang Province of China. This species lives in the water pits or ponds about 3 m diameter or adjacent grass thickets, subterranean holes and mud pits in the mountainous regions at elevation 1400 m a.s.l. The male frogs make vibrating calls on the last 10 days of March. Small tadpoles seen in the early April would dive to the bottom when disturbed. On the 9th April 1998, four gravid females, egg groups, and small tadpoles were found. Mating of females and males took place in the pond of imitated natural eco-breeding in Chengdu (Fei *et al.*, 1999). Small tadpoles were found in the last 10 days April. It took 60 days for the fertilized eggs to finish metamorphosis. Newly metamorphic frogs were 8–10 mm. Sympatric species of amphibians in the Beilun District during the surveys includes *Hynobius yiwuensis*, *Echinotriton chinhaiensis*, *Bufo gargarizans*, *Rana zhenhaiensis*, *Pelophylax nigromaculatus*, *Sylvirana latouchii*, *Fejervarya multistriata*.

**Eggs:** Animal pole brown, vegetative beige, egg diameter on average 1.0 mm, clutch size 400–657 eggs found in Beilun District, Ningbo City, Zhejiang province of China.

**Larvae:** At Gosner's stages 31–35 (early May; Gosner, 1960), tadpole's dorsum dark brown, front part lightly colored on both sides, upper and lower fins darkly colored on the margins, tail end thin and pointed, mouth dorso-

terminal, lower labial fold relatively wide, concaved in the middle with 4–5 papillae on the margin of the fold on each side of the middle, no labial teeth or horny beak, the snout-vent length of newly metamorphosed about 10.0 mm (Fei *et al.*, 1999).

#### 4. Discussion

Identifying *Microhyla* species only based on morphology is difficult and/or possibly problematic, because of their relatively high morphological similarity (Howlader *et al.*, 2015; Seshadri *et al.*, 2016). The molecular technology has made the taxonomic research, especially diagnosing cryptic taxa, more efficient and reliable (Khatiwada *et al.*, 2017; Matsui *et al.*, 2011). The specimens of *Microhyla beilunensis* sp. nov. has been ever recognized as *M. mixtura* (Huang *et al.*, 1990; Fei *et al.*, 1999, 2009, 2012). In this study, the phylogenetic data showed that the Beilun population formed a clade, and was sister to *M. mixtura*. Besides, the genetic distance between the new species and *M. mixtura* populations is 2.6% on 12S and 16S genes, equal to that between *M. malang* and *M. borneensis* (2.6%), and even greater than that between *M. mukhlesuri* and *M. fissipes* (2.4%), which conforms to the general species-level in amphibian sister taxa (Vences *et al.*, 2005). Additionally, both the unrooted tree and morphometric analyses could distinctly separate the new species from *M. mixtura*. Indeed, there are some subtle morphological characters between these two species after careful comparisons. All the evidences support that *Microhyla beilunensis* sp. nov. is a valid species.

The straight-line distance between the type locality of *Microhyla beilunensis* sp. nov. and the type locality of *M. mixtura* is approximately 1342 km. There are some high mountains (e.g. Daba Mountain and Dabie Mountain) and great river (e.g. Yangtze River) between the distributional ranges of the two species, which are often considered as the geographic barriers to block gene flow, facilitating genetic divergence and speciation (Chaves *et al.*, 2011; Craw *et al.*, 2008). *Microhyla beilunensis* sp. nov. prefers to the environment of standing water, and inhabits and breeds in hilly paddy fields, pools and grasslands (Fei *et al.*, 1999, 2009, 2012), possibly, indicating their limited dispersal ability. Therefore, the external barriers and inherent features have probably facilitated the genetic differentiation and speciation of this species from its sister species.

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## Appendix

**Table S1** Specimens examined in this study.

ID	Species	Voucher	Locality
1	<i>Microhyla mixtura</i>	CIBZMH2017061201	Yangxian County, Hanzhong City, Shaanxi Province, China
2	<i>Microhyla mixtura</i>	CIBZMH2017061202	Yangxian County, Hanzhong City, Shaanxi Province, China
3	<i>Microhyla mixtura</i>	CIBZMH2017061203	Yangxian County, Hanzhong City, Shaanxi Province, China
4	<i>Microhyla mixtura</i>	CIBZMH2017061204	Yangxian County, Hanzhong City, Shaanxi Province, China
5	<i>Microhyla mixtura</i>	CIBZMH2017061205	Yangxian County, Hanzhong City, Shaanxi Province, China
6	<i>Microhyla mixtura</i>	CIBZMH2017061206	Yangxian County, Hanzhong City, Shaanxi Province, China
7	<i>Microhyla mixtura</i>	CIBZMH2017061207	Yangxian County, Hanzhong City, Shaanxi Province, China
8	<i>Microhyla mixtura</i>	CIBZMH2017061208	Yangxian County, Hanzhong City, Shaanxi Province, China
9	<i>Microhyla mixtura</i>	CIB20170526001	Hua'e mountain, Wanyuan City, Sichuan Province, China
10	<i>Microhyla mixtura</i>	CIB20170526002	Hua'e mountain, Wanyuan City, Sichuan Province, China
11	<i>Microhyla mixtura</i>	CIB20170526003	Hua'e mountain, Wanyuan City, Sichuan Province, China
12	<i>Microhyla mixtura</i>	CIB20170526008	Hua'e mountain, Wanyuan City, Sichuan Province, China
13	<i>Microhyla mixtura</i>	CIB20170526009	Hua'e mountain, Wanyuan City, Sichuan Province, China
14	<i>Microhyla mixtura</i>	CIB20170526010	Hua'e mountain, Wanyuan City, Sichuan Province, China
15	<i>Microhyla mixtura</i>	CIB20170526011	Hua'e mountain, Wanyuan City, Sichuan Province, China
16	<i>Microhyla mixtura</i>	CIB20170526012	Hua'e mountain, Wanyuan City, Sichuan Province, China
17	<i>Microhyla mixtura</i>	CIB65684	Hua'e mountain, Wanyuan City, Sichuan Province, China
18	<i>Microhyla mixtura</i>	CIB65667	Hua'e mountain, Wanyuan City, Sichuan Province, China
19	<i>Microhyla beilunensis</i> sp. nov.	CIBBL001	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
20	<i>Microhyla beilunensis</i> sp. nov.	CIBBL002	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
21	<i>Microhyla beilunensis</i> sp. nov.	CIBBL003	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
22	<i>Microhyla beilunensis</i> sp. nov.	CIBBL004	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
23	<i>Microhyla beilunensis</i> sp. nov.	CIBBL005	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
24	<i>Microhyla beilunensis</i> sp. nov.	CIBBL006	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
25	<i>Microhyla beilunensis</i> sp. nov.	CIBBL007	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
26	<i>Microhyla beilunensis</i> sp. nov.	CIBA97001	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
27	<i>Microhyla beilunensis</i> sp. nov.	CIBA97002	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
28	<i>Microhyla beilunensis</i> sp. nov.	CIBA97003	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
29	<i>Microhyla beilunensis</i> sp. nov.	CIBA97004	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
30	<i>Microhyla beilunensis</i> sp. nov.	CIBA97005	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
31	<i>Microhyla beilunensis</i> sp. nov.	CIBA97006	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
32	<i>Microhyla beilunensis</i> sp. nov.	CIBA97007	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
33	<i>Microhyla beilunensis</i> sp. nov.	CIBA97008	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
34	<i>Microhyla beilunensis</i> sp. nov.	CIBA97009	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
35	<i>Microhyla beilunensis</i> sp. nov.	CIBA97010	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
36	<i>Microhyla beilunensis</i> sp. nov.	CIBA97011	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
37	<i>Microhyla beilunensis</i> sp. nov.	CIBA97012	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
38	<i>Microhyla beilunensis</i> sp. nov.	CIBA97013	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
39	<i>Microhyla beilunensis</i> sp. nov.	CIBA97014	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
40	<i>Microhyla beilunensis</i> sp. nov.	CIBA97015	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
41	<i>Microhyla beilunensis</i> sp. nov.	CIBA97016	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
42	<i>Microhyla beilunensis</i> sp. nov.	CIBA97017	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
43	<i>Microhyla beilunensis</i> sp. nov.	CIBA97018	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China

**(Continued Table S1)**

ID	Species	Voucher	Locality
44	<i>Microhyla beilunensis</i> sp. nov.	CIBA97019	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
45	<i>Microhyla beilunensis</i> sp. nov.	CIBA97020	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
46	<i>Microhyla beilunensis</i> sp. nov.	CIBA97021	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
47	<i>Microhyla beilunensis</i> sp. nov.	CIBA97022	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
48	<i>Microhyla beilunensis</i> sp. nov.	CIBA97023	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
49	<i>Microhyla beilunensis</i> sp. nov.	CIBA97024	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
50	<i>Microhyla beilunensis</i> sp. nov.	CIBA97025	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
51	<i>Microhyla beilunensis</i> sp. nov.	CIBB980059	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
52	<i>Microhyla beilunensis</i> sp. nov.	CIBB980060	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
53	<i>Microhyla beilunensis</i> sp. nov.	CIBB980061	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
54	<i>Microhyla beilunensis</i> sp. nov.	CIBB980062	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
55	<i>Microhyla beilunensis</i> sp. nov.	CIBB980063	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
56	<i>Microhyla beilunensis</i> sp. nov.	CIBB980064	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
57	<i>Microhyla beilunensis</i> sp. nov.	CIBB980065	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
58	<i>Microhyla beilunensis</i> sp. nov.	CIBB980066	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China
59	<i>Microhyla beilunensis</i> sp. nov.	CIBB980067	Chaiqiao Town, Beilun District, Ningbo City, Zhejiang Province, China



**Table S2** The results of Independent-Sample *T* tests on the morphometric characters between *Microhyla beilunensis* sp. nov. and *M. mixtura*. Morphometric characters were corrected by SVL. All measurements were given in millimeter (mm).

	<i>Microhyla beilunensis</i> sp. nov.		<i>M. mixtura</i> from the type locality		<i>t</i>	<i>P</i>
	Male ( <i>n</i> = 37)		Male ( <i>n</i> = 8)			
	Mean ± SD	Range	Mean ± SD	Range		
SVL	21.86 ± 1.16	19.08–23.73	22.16 ± 1.94	18.17–25.19	−0.59	NS
HL	0.29 ± 0.01	0.26–0.32	0.28 ± 0.01	0.26–0.31	1.99	NS
HW	0.35 ± 0.02	0.31–0.40	0.33 ± 0.02	0.30–0.35	3.00	**
SL	0.12 ± 0.01	0.11–0.14	0.13 ± 0.01	0.12–0.15	−2.38	*
IND	0.10 ± 0.01	0.08–0.14	0.09 ± 0.01	0.08–0.09	3.09	**
IOD	0.12 ± 0.01	0.09–0.15	0.13 ± 0.01	0.12–0.14	−2.18	*
UEW	0.08 ± 0.01	0.07–0.09	0.07 ± 0.00	0.06–0.08	5.10	***
ED	0.13 ± 0.01	0.09–0.14	0.12 ± 0.01	0.10–0.13	1.17	NS
LAHL	0.40 ± 0.02	0.37–0.45	0.43 ± 0.02	1.42–1.46	−5.30	***
LAW	0.07 ± 0.00	0.06–0.08	0.06 ± 0.00	0.05–0.06	4.45	***
HAL	0.25 ± 0.04	0.22–0.46	0.26 ± 0.01	0.24–0.27	−0.94	NS
HLL	1.61 ± 0.06	1.45–1.71	1.76 ± 0.04	1.70–1.83	−7.17	***
TL	0.54 ± 0.02	0.47–0.58	0.59 ± 0.02	0.57–0.63	−6.74	***
TW	0.16 ± 0.01	0.14–0.17	0.16 ± 0.01	0.14–0.17	0.34	NS
TFL	0.74 ± 0.02	0.70–0.80	0.83 ± 0.03	0.78–0.87	−9.714	***
FL	0.52 ± 0.02	0.47–0.57	0.58 ± 0.02	0.52–0.60	−6.62	***

Note: NS means  $P \geq 0.05$ , not significant; asterisks \*, \*\*, \*\*\* indicates  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively.

**Table S3** The results of Independent-Sample *T* tests on the morphometric characters between males and females of *Microhyla beilunensis* sp. nov.. Morphometric characters were corrected by SVL. All measurements were given in millimeter (mm).

	Male ( <i>n</i> = 37)		Female ( <i>n</i> = 4)		<i>t</i>	<i>p</i>
	Mean ± SD	Range	Mean ± SD	Range		
SVL	21.86 ± 1.16	19.08–23.73	27.58 ± 0.83	26.39–28.25	−9.58	***
HL	0.29 ± 0.01	0.26–0.32	0.27 ± 0.01	0.26–0.28	3.32	**
HW	0.35 ± 0.02	0.31–0.40	0.30 ± 0.02	0.27–0.33	4.60	***
SL	0.12 ± 0.01	0.11–0.14	0.11 ± 0.01	0.11–0.12	2.67	*
IND	0.10 ± 0.01	0.08–0.14	0.08 ± 0.00	0.08–0.09	2.92	**
IOD	0.12 ± 0.01	0.09–0.15	0.10 ± 0.01	0.10–0.11	1.91	NS
UEW	0.08 ± 0.01	0.07–0.09	0.07 ± 0.01	0.06–0.08	2.03	NS
ED	0.13 ± 0.01	0.09–0.14	0.11 ± 0.01	0.11–0.12	3.06	**
LAHL	0.40 ± 0.02	0.37–0.45	0.36 ± 0.00	0.36–0.37	4.54	***
LAW	0.07 ± 0.00	0.06–0.08	0.05 ± 0.00	0.05–0.05	5.91	***
HAL	0.25 ± 0.04	0.22–0.46	0.22 ± 0.01	0.20–0.24	1.34	NS
HLL	1.61 ± 0.06	1.45–1.71	1.49 ± 0.03	1.44–1.51	3.96	***
TL	0.54 ± 0.02	0.47–0.58	0.50 ± 0.02	0.49–0.52	3.04	***
TW	0.16 ± 0.01	0.14–0.17	0.14 ± 0.01	0.13–0.15	3.12	***
TFL	0.74 ± 0.02	0.70–0.80	0.71 ± 0.02	0.68–0.73	2.96	***
FL	0.52 ± 0.02	0.47–0.57	0.48 ± 0.01	0.47–0.49	4.69	***

Note: NS means  $P \geq 0.05$ , not significant; asterisks \*, \*\*, \*\*\* indicates  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively.